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Adenovirus E1B 19 kDa and Bcl-2 Proteins Interact with a Common Set of Cellular Proteins

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Summary

Adenovirus E1B 19 kDa protein protects against cell death induced by viral infection and certain external stimuli. The Bcl-2 protein can functionally substitute for the E1B 19 kDa protein. To identify cellular targets for the 19 kDa protein, we used the two-hybrid screen in yeast. We have isolated cDNAs for three different proteins, designated Nip1, Nip2, and Nip3, that interact with the 19 kDa protein. Mutational analysis indicates that these proteins do not associate with 19 kDa mutants defective in suppression of cell death, suggesting a correlation between interaction of these proteins and suppression of cell death. These proteins also associate with discrete sequence motifs in the Bcl-2 protein that are homologous to motifs of the 19 kDa protein. Our results suggest that two diverse proteins, the E1B 19 kDa and the Bcl-2 proteins, promote cell survival through interaction with a common set of cellular proteins.

Introduction

The 19 kDa protein coded by the adenovirus E1B region confers a survival function in adenovirus-infected cells and prevents premature cell death. Adenovirus mutants defective in the *19K* gene produce large clear plaques on infected cell monolayers (Chinnadurai, 1983). Several of these mutants induce an enhanced cytopathic effect in infected cells resulting in cellular destruction (Takemori et al., 1968, 1984; Subramanian et al., 1984a) as well as fragmentation of cellular and viral DNA (Ezoe et al., 1981; Subramanian et al., 1984b; Pilder et al., 1984; White et al., 1984a; D'Halluin et al., 1985). The DNA fragmentation induced by *19K* mutants is reminiscent of that observed during apoptosis (Wyllie, 1980). Although it has not yet been determined whether DNA fragmentation induced by *19K* mutants occurs by an apoptotic mechanism, it is clear that the 19 kDa protein protects against a cell death program induced by viral infection, thus facilitating efficient virus replication.

In addition, the 19 kDa protein suppresses the cytotoxic effects of certain external stimuli such as the tumor necrosis factor α (Gooding et al., 1991; White et al., 1992) and anti-Fas antibody (Hashimoto et al., 1991). Both these agents cause cell death through apoptosis (Laster et al., 1988; Itoh et al., 1991). Similarly, the 19 kDa protein protects cells against the effects of DNA-damaging agents such as the anti-cancer drug cisplatin (Subramanian et al., 1993) and ultraviolet radiation (Tarodi et al., 1993). Cisplatin (Sorenson and Eastman, 1988) and ultraviolet radiation (reviewed by Williams, 1991) induce cell death through a p53-dependent apoptotic pathway (Lane, 1993). The 19 kDa protein can also efficiently suppress cell death induced by overexpression of p53 (Debbas and White, 1993). Thus, the 19 kDa protein provides a survival function in virus-infected cells and also protects cells against certain other death-inducing stimuli.

The survival function provided by E1B *19K* is similar to the activity of the cellular protooncogene *Bcl-2*. The *Bcl-2* oncogene was isolated from a follicular lymphoma (Tsujimoto et al., 1985; Bakhshi et al., 1985; Cleary and Sklar, 1985) and has been shown to enhance the survival of hematopoietic B and T cells by blocking apoptosis (Vaux et al., 1988; Hockenbery et al., 1990; Sentman et al., 1991; Strasser et al., 1991). In addition, overexpression of Bcl-2 protein inhibits apoptosis induced by exposure to diverse stimuli possibly through different pathways. Although the effect of 19 kDa protein expression on cell death induced by diverse stimuli has not been extensively examined, the Bcl-2 protein can clearly substitute for the 19 kDa protein during adenovirus infection. Characteristic fragmentation of cellular DNA induced by infection with adenovirus type 2 (Ad2) *19K* mutants is efficiently suppressed in cells ectopically expressing the human Bcl-2 protein (Tarodi et al., 1993). Similarly, expression of Bcl-2 by an Ad2–Bcl-2 recombinant virus (Ad–Bcl2) can fully substitute for the *19K* function. The Ad–Bcl2 virus does not induce DNA fragmentation in infected cells and forms small plaques on cell monolayers (T. S. et al., submitted). Rao et al. (1992) have reported that *Bcl-2* can substitute for *19K* in transformation of primary rat kidney cells in cooperation with E1A. Although these results do not clarify whether the 19 kDa and Bcl-2 proteins function by similar mechanisms, they indicate that these two proteins can provide cell survival function against certain specific stimuli.

The mechanism by which the *19K* gene and the *Bcl-2* protooncogene protect against cell death is not known. These proteins may mediate cell survival by interacting with certain cellular proteins. To identify the cellular proteins that interact with the E1B 19 kDa protein, we used the two-hybrid protein interaction cloning system in the yeast Saccharomyces cerevisiae (Fields and Song, 1989). Here we report the cloning and characterization of three human cDNAs for proteins that interact with discrete domains of the E1B 19 kDa protein involved in suppression of cell death. These proteins also interact with the Bcl-2 protein through similar sequence motifs, suggesting that the 19 kDa and Bcl-2 proteins may share certain biochemical pathways and promote cell survival through interaction with a common set of cellular proteins.

Results

Isolation of cDNA Clones for 19 kDa-Interacting Proteins

For two-hybrid screening, we used a fusion protein bait

*These authors made equal contributions.

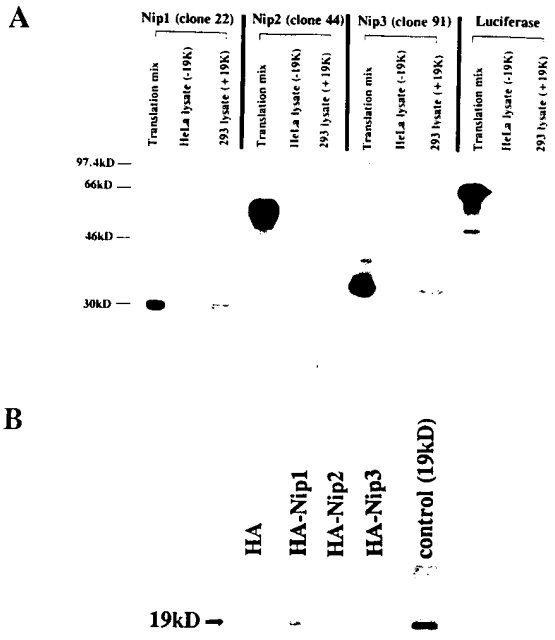


Figure 1. Interaction of Cellular Proteins with the 19 kDa Protein

(A) Immunoprecipitation of in vitro labeled cellular proteins added to extracts of HeLa or 293 cells. Proteins were immunoprecipitated with 19 kDa antiserum and analyzed on 15% SDS–PAGE.

(B) Immunoblot of 19 kDa protein. HA immunoprecipitates from BSC40 cells transfected with pTM plasmids were analyzed for the presence of coprecipitating 19 kDa protein by probing Western blots with 19 kDa antibody followed by chemiluminescent detection.

consisting of the yeast GAL4 DNA-binding domain (amino acids 1–147) and E1B 19 kDa protein expressed from a yeast shuttle vector (pMA424-19kDa or pAS-19kDa). Plasmids expressing the 19 kDa bait and a human cDNA expression library (pAct) tagged with the GAL4 activation domain (Durfee et al., 1993) were used for simultaneous transformation of two yeast indicator strains, GGY1::171 (Gill and Ptashne, 1987) or Y153 (Durfee et al., 1993). About 10^5 transformants of each strain were screened for the activation of the reporter genes, and four strongly positive clones, judged by the level of expression of the reporter *lacZ*, were chosen for further analysis. These clones were tested in the two-hybrid screen with the 19 kDa bait or with the GAL4 DNA-binding domain vectors (pMA424 or pAS1). All four clones reacted positively only with the 19 kDa bait and not with the respective vectors. Three of these clones (numbers 22, 44, and 91) were found to be defective in interaction with *19K* mutant baits of interest. The characterization of these three clones is reported here. To determine the specificity of the two-hybrid interaction, clones 22, 44, and 91 were further tested against a battery of nine different heterologous protein baits that included various HIV and HTLV regulatory proteins, yeast SNF1, human lamin, adenovirus E1A exon 2, and E3 11.6 kDa protein as well as the baculovirus 35 kDa protein. All three clones were negative against all the heterologous protein baits tested, indicating that the proteins coded by these clones specifically interact with the 19 kDa protein (data not shown).

Interactions In Vitro and In Vivo

To substantiate the interactions observed in yeast, we carried out an in vitro protein binding assay. The proteins coded by the various cDNA clones were prepared by in vitro transcription and translation. ^{35}S-labeled proteins coded by clones 22, 44, and 91 were incubated with protein extracts prepared from human 293 cells that expressed the 19 kDa protein or with extracts of HeLa cells that did not express any adenovirus proteins. The interaction of the labeled proteins with the 19 kDa protein was then analyzed by immunoprecipitation with an antipeptide 19 kDa antiserum. As seen in Figure 1A, exogenously added ^{35}S-labeled proteins produced from these three clones were readily precipitated with 19 kDa antibodies in the presence of 293 cell extracts. Under the same binding conditions, ^{35}S-labeled luciferase (control) was not precipitated. No significant amounts of the labeled proteins were precipitated with 19 kDa antibodies from HeLa cell extracts (Figure 1A). In all cases, immunoprecipitations with normal rabbit serum did not precipitate any detectable levels of these proteins (data not shown). These results indicate that the proteins coded by clones 22, 44, and 91 (hereafter referred to as 19 kDa–interacting proteins 1, 2, and 3 [Nip1, Nip2, and Nip3], respectively) specifically interact with the 19 kDa protein either directly or indirectly via other cellular proteins and substantiate the results obtained with the two-hybrid screens.

To confirm the interaction of the Nip proteins with the 19 kDa protein in mammalian cells, we transfected BSC40 cells with plasmid vectors expressing the 19 kDa or various Nip proteins under the transcriptional control of the T7 promoter in a mammalian expression vector, pTM1 (Moss et al., 1990). These cells were infected with the recombinant vaccinia virus vTF7-3, expressing the T7 RNA polymerase gene (Fuerst et al., 1986), to induce the expression of proteins from the various pTM1 plasmids. The Nip proteins were tagged with an epitope corresponding to a 9 amino acid region of the hemagglutinin (HA) protein of influenza virus (Field et al., 1988). The pTM1 vector expressing only the HA epitope was used as a control. Cells were labeled in vivo with [^{35}S]methionine–cysteine mixture and lysed, and the lysates were divided and subjected to immunoprecipitation using either a monoclonal HA antibody or a 19 kDa antiserum. The precipitated proteins were analyzed by SDS–PAGE to determine that comparable levels of the 19 kDa protein were present in each sample. The HA immunoprecipitates were separated by SDS–PAGE, blotted onto nitrocellulose, and probed with the 19 kDa antibody to detect the presence of coprecipitating 19 kDa protein. A horseradish peroxidase–enhanced chemiluminescent detection system (ECL) was used to visualize bound antibodies. The 19 kDa protein was not detected in HA immunoprecipitates from cells transfected with the pTM1–HA vector and pTM1–19kDa, but was clearly seen coprecipitating with HA-tagged Nip1, Nip2, and Nip3 (Figure 1B). A lane showing the 19 kDa protein immunoprecipitated with the 19 kDa antibody (from cells transfected with

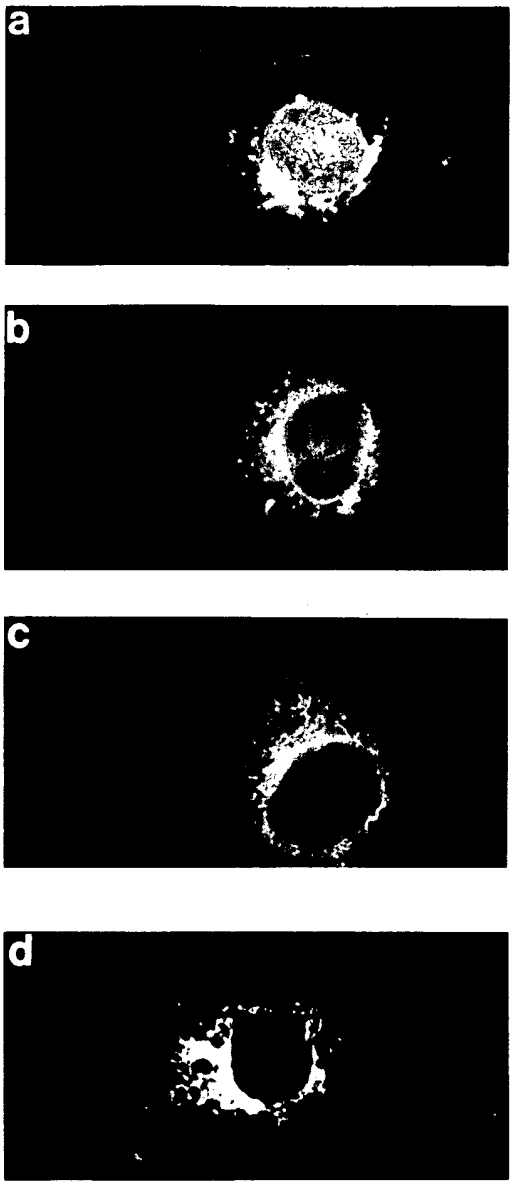


Figure 2. Subcellular Localization of the 19 kDa and Nip Proteins

COS7 cells were transfected with plasmids expressing the 19 kDa protein (a), Nip1 (b), Nip2 (c), or Nip3 (d). The transfected cells were stained with a 19 kDa antibody or HA monoclonal antibody and visualized with rhodamine (19 kDa) or fluorescein (HA).

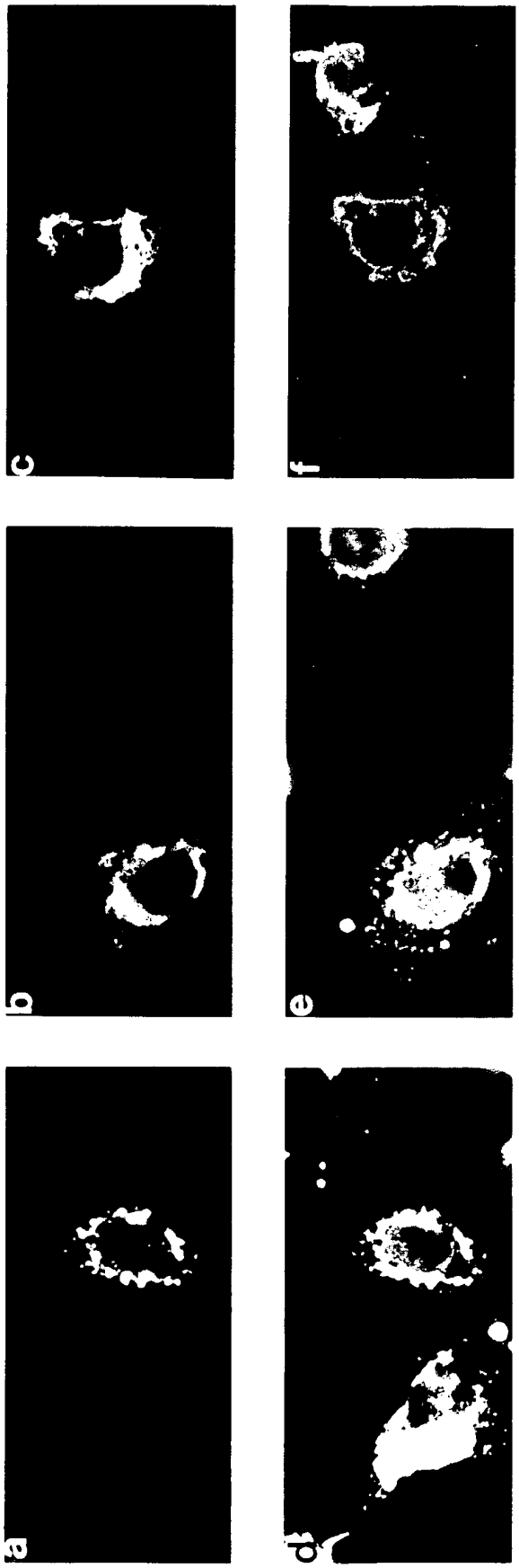
pTM1–19kDa) was included on the blot as a positive control. In experiments using a 19 kDa mutant (pTM1–19kDa(90-6)), no detectable coprecipitation was observed, providing additional support for the specificity of these interactions (data not shown). These results indicate that the 19 kDa protein interacts specifically with each of the Nip proteins in an in vivo coimmunoprecipitation assay.

Subcellular Localization of Nips

To examine the subcellular localization of the Nips and to determine whether these proteins colocalize with the 19 kDa protein inside cells, we transfected COS7 cells with plasmids expressing each Nip tagged with the HA epitope, either alone or with a 19 kDa-expressing plasmid, and analyzed the transfected cells by indirect immunofluorescence. A monoclonal HA antibody and a 19 kDa antiserum visualized with fluorescein and rhodamine-tagged secondary antibodies, respectively, were used to detect the proteins in transfected cells. The location of each Nip was examined in the absence (Figure 2) or presence (Figure 3) of the 19 kDa protein. As expected (White et al., 1984b), the 19 kDa protein was primarily expressed at the nuclear envelope/endoplasmic reticulum region (Figure 2a). Nip1 (clone 22) and Nip2 (clone 44) also appear to be primarily concentrated on the nuclear envelope region in addition to other cytoplasmic structures (Figures 2b and 2c). Nip3 has a different pattern of localization (Figure 2d). It has a punctate pattern resembling the patterns exhibited by mitochondria (Alberts et al., 1989). In cells coexpressing Nip1 (Figures 3a and 3d), Nip2 (Figures 3b and 3c), or Nip3 (Figure 3c and 3f) and the 19 kDa protein, both the 19 kDa (Figures 3a–3c) and each of the cellular proteins (Figures 3d–3f) colocalized primarily at the nuclear envelope region, thus displaying a pattern identical to that observed for the 19 kDa protein alone. This involves a marked shift in the localization pattern of Nip3; the characteristic punctate localization pattern (see Figure 2d) was not observed in the presence of the 19 kDa protein (Figure 3f). These results suggest that the 19 kDa protein and the various cellular proteins exist and function primarily at the nuclear envelope region as complexes with the 19 kDa protein.

Mutational Analysis of Nip-19 kDa Interactions

We have previously characterized the ability of a series of *19K* mutants to protect against cell death induced by the DNA-damaging agent cisplatin and to cooperate with *E1A* in transformation of primary cells (Subramanian et al., 1993). The 19 kDa coding regions of these mutants were cloned in the pMA424 vector and tested for interactions with Nip1, Nip2, and Nip3 in the two-hybrid assay. Interactions were assessed by examining *lacZ* expression by the X-Gal filter blue/white color assay as well as by a quantitative o-nitrophenyl-β-D-galactopyranoside (ONPG)-based β-galactosidase assay (Table 1). All three 19 kDa–interacting proteins were negative for interaction with mutants 50-1 (substitutions for amino acids 50–51) and 90-6 (deletion of amino acids 90–96). Nip1 and Nip2 interacted at a reduced level with mutant 123-4 (substitutions for residues 123–124), but actually interacted more efficiently with the *19K* mutant 75-6 (substitutions for residues 75–76) than with wild-type *19K*. Nip3 exhibited a similar pattern of interaction; however, the overall level of expression of *lacZ* was lower. These studies identify two regions of the 19 kDa protein marked by mutants 50-1 and 90-6 critical for interaction with the Nip proteins. In addition, sequences around residues 123–124 (mutant 123-4) may also influence these interactions. The pattern of interaction of the three Nips with the 19 kDa protein is illustrated in Figure 4. All the *19K* mutants have been shown to make stable proteins in mammalian cells and as GAL4(1–147)–19 kDa fusion proteins in yeast (results not shown). Thus, the *19K*



mutants 50-1 and 90-6 defective for suppression of cell death are also defective for interaction with the Nip proteins. The mutant 123-4, which is also defective in suppression of cell death, interacts poorly with these proteins. These results suggest a correlation between interaction of the Nip proteins with the 19 kDa protein and suppression of cell death.

Interaction of Nips with Bcl-2

Since *Bcl-2* can functionally substitute for *19K* during adenovirus replication, we asked whether the 19 kDa–interacting cellular proteins also interact with the Bcl-2 protein. First, we carried out the two-hybrid analysis using a pMA424-based bait expressing the human Bcl-2 protein. Yeast cells (GGY1::171) were transformed either with the pMA424–19K bait or with the pMA424–Bcl-2 bait along with each of the activation domain–tagged cDNA clones. Analysis of *lacZ* expression (Table 2) revealed that all three clones interacted with the Bcl-2 bait. Nip1 and Nip3 interacted more efficiently with the Bcl-2 bait compared with the 19 kDa bait. The interaction of Nip2 was slightly lower with Bcl-2 than with 19 kDa. To substantiate further the interaction between the Bcl-2 protein and the cellular Nips, we carried out an in vitro immunoprecipitation assay as described earlier. Proteins coded by the three cDNA clones were prepared by in vitro transcription and translation and incubated with cell extracts prepared from CHO cells or CHO cells infected with an adenovirus recombinant that overexpresses the human Bcl-2 protein (Ad–Bcl2). The proteins were immunoprecipitated with a hamster Bcl-2 monoclonal antibody and analyzed by SDS–PAGE (Figure 5A). All three Nip proteins were readily precipitated from extracts prepared from cells infected with the Ad–Bcl2 virus. In contrast, there was no significant amount of the Nip1 and Nip3 proteins precipitated from uninfected cells. Small but variable amounts of Nip2 were precipitated from uninfected cells, suggesting that this protein may be precipitated nonspecifically with the Bcl-2 antibody at low levels. However, the interaction between Bcl-2 protein and Nip2 appears to be specific, on the basis the two-hybrid analysis in yeast and the enhanced levels of Nip2 precipitated from Ad–Bcl2-infected cells compared with uninfected cells in the in vitro assay.

To substantiate further the interaction of each Nip with Bcl-2, we carried out immunoprecipitation studies similar to those described for the 19 kDa protein (see Figure 1B). BSC40 cells that were infected with vTF7–3 and cotransfected with pTM1-based plasmids expressing Bcl-2 and one of the HA-tagged Nips were labeled, lysed, and analyzed essentially as described above. Comparable levels

Figure 3. Colocalization of the 19 kDa and Nip Proteins

COS7 cells were transfected with plasmids expressing the 19 kDa protein along with plasmids expressing HA-tagged Nip1 (a and d), Nip2 (b and e), or Nip3 (c and f). Cells were stained with both 19 kDa antibody (visualized with rhodamine) and HA monoclonal antibody (visualized with fluorescein) and photographed using filters specific for rhodamine (a–c) or fluorescein (d–f). The same fields of cells were photographed under both filters.

Table 1. Interaction of Cellular Proteins with *19K* Mutants

Mutant	Cell Death Suppression	Relative Level of *lacZ* Expression					
		Nip1		Nip2		Nip3	
		X-Gal	ONPG	X-Gal	ONPG	X-Gal	ONPG
Wild type	+	B	1	B	1	B	1
14-5	(+)	B	0.61	B	0.55	LB	0.16
29-0	+	B	0.33	B	0.32	B	0.19
50-1	−	W	<0.01	W	0.09	W	0.01
75-6	+	B	1.72	B	1.60	B	0.22
90-6	−	W	<0.01	W	0.04	W	0.01
123-4	−	W	0.05	LB	0.24	LB	0.10
146-E	+	B	0.87	B	0.98	B	0.28

The protein interaction studies were carried out in yeast strain GGY1::171. Results of X-Gal staining are indicated as B for blue, W for white, or LB for light blue. The various *19K* mutants and their effects on cell death are described in Subramanian et al. (1993). (+) indicates positive at reduced levels. β-Galactosidase activities (Rose et al., 1990) were determined from three to four independent colonies selected at random.

of Bcl-2 protein were present in each sample, as determined by immunoprecipitation using the Bcl-2 monoclonal antibody. Each sample was subjected to immunoprecipitation using the HA monoclonal antibody; the precipitated proteins were separated by SDS–PAGE, blotted, and probed for the presence of Bcl-2. Bcl-2 was clearly coprecipitated with HA–Nip1, HA–Nip2, and HA–Nip3 (Figure 5B). A control lane showing precipitated Bcl-2 protein was included on the blot. Thus, using an in vivo coimmunoprecipitation assay, we have demonstrated a specific interaction between each Nip protein and Bcl-2. These results, together with the in vitro immunoprecipitation data, indicate that the three Nips interact with the Bcl-2 as well as with the 19 kDa proteins.

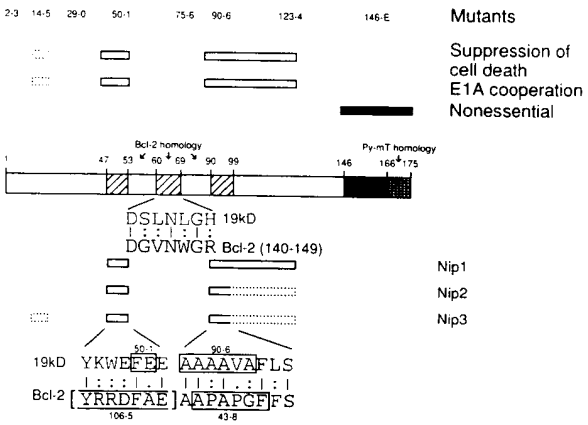


Figure 4. Functional Organization of the 19 kDa Protein

The 19 kDa sequences involved in suppression of cisplatin-induced cell death and E1A-cooperative transformation are based on Subramanian et al. (1993). Solid bars indicate strong effects of various mutants on 19 kDa functions and interaction with Nip proteins; bars with dashed lines indicate weak effects. The sequence similarity between the 19 kDa and Bcl-2 proteins and the mutants that map within these sequences are shown at the bottom of the figure. The human Bcl-2 protein used in our studies is a mutant form isolated from a human lymphoma and contains a Phe (instead of Ile) residue at position 48 and two other mutations (Seto et al., 1988). Identical amino acids are indicated by solid lines, similar amino acids by colons, and distantly related amino acids by single dots.

Interaction of Nips with Bcl-2 Mutants

The observation that the various Nip proteins also interact with the Bcl-2 protein is somewhat surprising, since the primary amino acid sequence of the 19 kDa protein does not appear to be homologous to Bcl-2 and related proteins (Williams and Smith, 1993). We searched the Bcl-2 protein for regions of homology corresponding to the regions of 19 kDa protein required for interaction with the Nip proteins. We tentatively identified three regions of homology between the amino acid sequences encompassing the *19K* mutants (50-1 and 90-6) and Bcl-2. On the basis of these results, we constructed three different deletion mutants within the Bcl-2-coding region. These mutants were cloned in pMA424 and used in the two-hybrid assays. Interaction was determined by *lacZ* expression assays (Table 3). Mutants 42-8 (residues 42–48) and 106-5 (residues 106–115) were defective for interaction with all three clones, while mutant 80-6 (residues 80–86) was not. Since the amino acid sequences of Bcl-2 deleted in mutants 42-8 and 106-5 appear to be homologous to sequences around *19K* mutants 90-6 and 50-1, respectively, it appears that the 19 kDa–interacting proteins recognize bipartite sequence motifs common to both the 19 kDa and Bcl-2 proteins (see Figure 4).

Sequence Analysis of cDNAs

The DNA sequences of the three cDNA clones were determined, and the reading frame in relation to the GAL4 activation domain was established. We obtained 5′-upstream sequences for each cDNA clone by reverse transcription

Table 2. Interaction of Nip proteins with Bcl-2

Protein	Relative Level of *lacZ* Expression			
	19 kDa		Bcl-2	
	X-Gal	ONPG	X-Gal	ONPG
Nip1	B	1	B	1.8
Nip2	B	1	B	0.7
Nip3	B	1	B	1.5

Relative interaction is based on β-galactosidase activity in cells expressing the respective clones and the *19K* bait. B, blue.

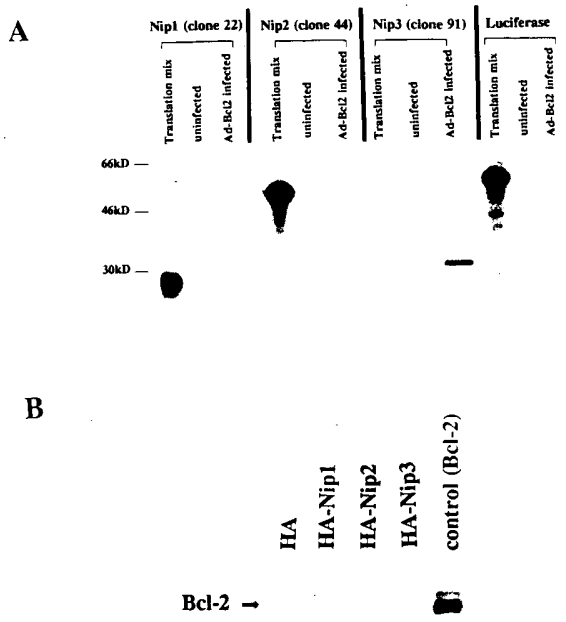


Figure 5. Interaction of Nip Proteins with the Bcl-2 Protein

(A) Immunoprecipitation of in vitro labeled Nip proteins added to cell extracts of CHO cells infected with Ad–Bcl2 (25 PFU/cell) or uninfected CHO cells. Proteins were immunoprecipitated with Bcl-2 antibody and analyzed on 15% SDS–PAGE.

(B) Immunoblot of Bcl-2. HA-immunoprecipitates from BSC40 cells transfected with pTM plasmids were analyzed for the presence of co-precipitating Bcl-2 protein by probing Western blots with Bcl-2 antibody as in Figure 1B.

of poly(A) containing RNA prepared from HeLa cells or human placenta, and polymerase chain reaction amplification using two nested primers (5′ rapid amplification of cDNA ends [RACE]). The RACE analysis provided additional DNA sequences corresponding to three codons, including an ATG codon for clone 22 (Nip1), while extensive analysis did not provide additional protein-coding sequences for cDNA clones 44 (Nip2) and 91 (Nip3). The coding region of clone 44 at the 3′ end (17 amino acids) was obtained by 3′ RACE analysis and from an independent cDNA clone from a B cell cDNA library. The amino acid sequences of Nip1, Nip2, and Nip3 based on DNA sequences are shown in Figure 6. These amino acid sequences were further analyzed to determine the similarity to other sequences in the various data banks (GenBank, PIR, and SwissProt) using the FASTA, TFASTA (UWGCG package), and BLAST (Altschul et al., 1990) (National Center for Biotechnology Information) algorithms. The sequences were also analyzed by the PROSITE program to identify functional sequence motifs.

Nip1 is a 228 amino acid protein and contains a putative membrane-spanning hydrophobic domain. The presence of this membrane-spanning domain could enable the protein to form a stable association with cellular membranes and may account for the observed localization of the protein to the nuclear envelope/endoplasmic reticulum region of the cell (see Figure 2b). Nip1 is not significantly homologous to other known proteins. However, a 59 to 83 amino acid region of Nip1 shows some homology (29%–36% identity and 55%–60% similarity) to a conserved region (Figure 7A) within the catalytic domain of three mammalian (rat, mouse, and cow) 3′-5′-cyclic nucleotide phosphodiesterases (Bentley et al., 1992; Polli and Kincaid, 1992; Repaske et al., 1992).

Nip2 is a 315 amino acid protein. A 126 amino acid region of Nip2 shares significant homology (47% identity and 66% similarity; Figure 7C) to the human GTPase-activating protein RhoGAP (Barfod et al., 1993; Lancaster et al., 1994). The homology between Nip2 and RhoGAP is located upstream of the functional domain of RhoGAP. The Nip2 sequence also contains a putative Ca^{2+}-binding motif (Figure 7B). Even though Nip2 does not contain a membrane-spanning hydrophobic domain, indirect immunofluorescence analysis indicates that it is associated with cytoplasmic structures.

Nip3 is a novel 194 amino acid protein and contains a presumptive membrane-spanning hydrophobic domain. The cDNA sequence is highly homologous to a rat cDNA clone that encodes the rat brain calbindin-D protein (Hunziker and Schrickel, 1988). A smaller cDNA clone isolated from human fetal brain (Adams et al., 1992) is identical to sequences within the Nip3 coding region. cDNA clone 91 (Nip3) and the fetal brain cDNA do not contain the coding sequences for calbindin. Similarly, two other cDNA clones containing Nip3 sequences that we have isolated from a human B cell cDNA library do not contain the calbindin-coding sequences. An extensive search for additional sequences in DNA amplified by 5′ RACE of human placental RNA with primers specific for clone 91 did not yield additional 5′ sequences corresponding to calbindin-coding sequences. Thus, it appears that Nip3 is coded by a unique

Table 3. Interaction of Nip Proteins with *Bcl-2* Mutants

	Relative Level of *lacZ* Expression					
	Nip1		Nip2		Nip3	
Mutant	X-Gal	ONPG	X-Gal	ONPG	X-Gal	ONPG
Wild type	B	1.0	B	1.0	B	1.0
42-8	W	0.16	W	0.09	W	0.19
80-6	B	1.21	B	0.80	B	2.87
106-5	W	0.01	W	0.06	W	0.11

Relative interaction is based on β-galactosidase activity in cells expressing the respective clones and the *Bcl-2* bait. B, blue; W, white.

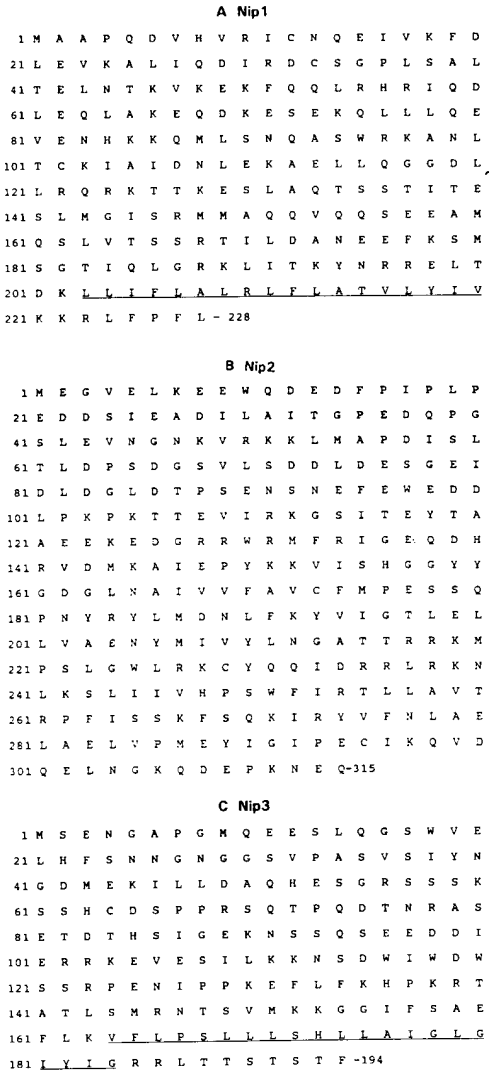


Figure 6. Amino Acid Sequences of the 19 kDa–Interacting Cellular Proteins

The putative transmembrane domains of Nip1 and Nip3 are underlined. Nip2 amino acid 290 is V not I.

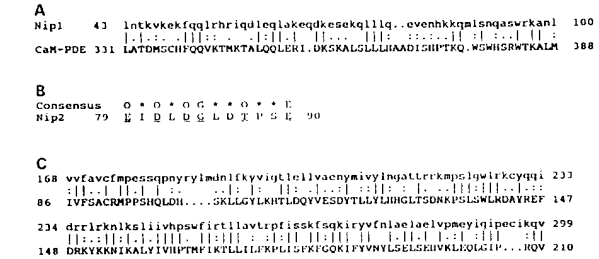


Figure 7. Sequence Homologies

(A) Homology between Nip1 and the catalytic domain of rat PDE. Similar homologies are also observed with mouse and cattle PDEs.

(B) Putative Ca^{2+}-binding motif of Nip2. O, oxygen-containing residues; asterisk, nonconserved residues; underlined residues conform to consensus residues.

(C) Homology between Nip2 and human RhoGAP.

mRNA in human cells. This notion is in agreement with the observation that rat calbindin cDNA hybridizes to multiple mRNA species from human brain (Iacopino and Christakos, 1990). All three cellular proteins described here contain PEST sequences, suggesting that these proteins may be degraded rapidly and expressed in a stage-specific manner (Rogers et al., 1986).

Discussion

We identified and isolated cDNA clones for three novel cellular proteins, Nip1, Nip2, and Nip3, that interact with the adenovirus E1B 19 kDa protein. We used different approaches to demonstrate that these proteins interact specifically with the 19 kDa protein. When screened in yeast, they interact with the 19 kDa protein and not with a battery of heterologous proteins. These proteins are coprecipitable with the 19 kDa protein in in vitro and in vivo immunoprecipitation assays. The Nips also colocalize with the 19 kDa protein in transfected cells. Further, specific mutations within the 19 kDa coding region abolish their ability to interact with the 19 kDa protein.

It appears that the interaction of the Nip proteins with the 19 kDa protein may be related to the ability of the 19 kDa protein to provide a cell survival function, since *19K* mutants defective in this activity do not interact with these proteins. This view is strengthened by our results demonstrating that these 19 kDa–interacting proteins also interact with the Bcl-2 protein. A fourth 19 kDa–interacting cellular protein (clone 85), which interacts with 19 kDa sequences not involved in suppression of cell death, does not interact with the Bcl-2 protein (data not shown). We have also observed that the Nip proteins can interact with the BHRF1 protein of Epstein–Barr virus (results not shown). BHRF1 shares significant homology with the Bcl-2 protein (Williams and Smith, 1993) and protects against cell death induced by certain apoptosis-inducing stimuli, such as serum depletion and DNA-damaging agents (Henderson et al., 1993; Tarodi et al., 1994).

The interaction of the Nip proteins with three different viral and cellular proteins involved in suppression of cell death is remarkable, since the Bcl-2 and BHRF1 proteins do not share significant overall sequence homology with the 19 kDa protein. However, the bipartite 19 kDa sequence motif involved in interacting with the Nip proteins is also shared by the Bcl-2 protein (Figure 4). Mutations in the homologous sequences of Bcl-2 abolish the interaction of these proteins with Bcl-2. Thus, it appears that Nip1, Nip2, and Nip3 interact with the 19 kDa and Bcl-2 proteins through homologous sequence motifs. Based on homology with the 19 kDa sequences involved in suppression of cell death and interaction with these proteins, we believe that the corresponding Bcl-2 sequences are also involved in suppression of cell death. Although sequences homologous to the 19 kDa motifs are present in the BHRF1 protein, the role of these sequences in interaction with cellular proteins has yet to be determined. It is interesting to note that the two domains of the 19 kDa and Bcl-2 proteins

required for interaction with the Nip proteins are absent in Bax, a homolog of Bcl-2 (Oltvai et al., 1993). The absence of these two domains in Bax, which has a cell death–promoting activity, supports the observation that these two domains are important for mediating cell survival by the 19 kDa and Bcl-2 proteins.

The mechanism by which the 19 kDa and Bcl-2 proteins promote cell survival is not known. The 19 kDa and Bcl-2 proteins may suppress cell death by antagonizing the activity of the 19 kDa–interacting proteins. Such a mechanism has been proposed for suppression of programmed cell death in the nematode Caenorhabditis elegans, where apoptotic cell death is inhibited by a survival-promoting gene designated *ced-9* (Hengartner et al., 1992). The *ced-9* gene antagonizes the activity of two nematode cell death–inducing genes, *ced-3* and *ced-4*, which are activated during programmed cell death (Yuan and Horvitz, 1990). In mammalian cells, one of the mechanisms by which Bcl-2 inhibits apoptosis may involve Bax–Bcl-2 heterodimerization (Oltvai et al., 1993).

Since the 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins, it appears that these proteins and possibly the BHRF1 protein may use a similar mechanism to suppress cell death induced by a specific set of stimuli. The 19 kDa protein is predominantly localized in the nuclear envelope/endoplasmic reticulum region. Similarly, Bcl-2 is also predominantly localized on the nuclear envelope/endoplasmic reticulum region (Alnemri et al., 1992; Jacobson et al., 1993), although localization on the inner mitochondrial membrane has also been reported (Hockenbery et al., 1990). Our immunofluorescence analysis shows that Nip1 and Nip2 localize at the nuclear envelope/endoplasmic reticulum regions, while Nip3 localizes in mitochondria and other cytoplasmic membrane structures. In the presence of the 19 kDa protein (Figure 3) or Bcl-2 (data not shown), each Nip colocalizes with the 19 kDa protein or Bcl-2 at the nuclear envelope region. Thus, the nuclear envelope/endoplasmic reticulum region may be an important subcellular area where the survival-promoting proteins such as the 19 kDa and Bcl-2 proteins may function in association with other cellular proteins. It is interesting that the Nip proteins interact with the same 19 kDa sequences, suggesting that multiple proteins may act in concert as a complex to suppress cell death. This model assumes that these proteins (or their homologs) may exist in yeast.

The homology between Nip2 and RhoGAP raises the possibility that the signal transduction pathway may play a role in counteracting the cell death–inducing stimuli. In this context, it has recently been reported that Bcl-2 associates with R-Ras (Fernandez-Sarabia and Bischoff, 1993), a member of the Ras superfamily. Since the homology between Nip2 and RhoGAP does not include the GTPase activation or SH3 domains of RhoGAP (Barfod et al., 1993, Lancaster et al., 1994), it unlikely that Nip2 is a new RhoGAP protein. It is possible that Nip2 may modulate the activity of RhoGAP through alternate mechanisms such as formation of RhoGAP–Nip2 heterodimers. Active RhoGAP protein (29 kDa) from human tissues appears to be processed from a larger 50 kDa precursor (Lancaster et al., 1994). Nip2 might affect the activity of RhoGAP by interfering with the processing of RhoGAP. It is interesting to note that Nip2 has a putative Ca^{2+}-binding motif. Since intracellular Ca^{2+} appears to be an important mediator of apoptotic cell death, it is possible that the activity of Nip2 may be modulated by Ca^{2+}. We could envision a model where interactions between the 19 kDa protein and Nip2 could remove its influence on RhoGAP activity, thus affecting Rho and potentially other members of the Rho family such as Rac and CDC42.

It is intriguing that Nip1 shares some homology with the catalytic domain of three mammalian calcium/calmodulin-dependent cyclic nucleotide phosphodiesterases (PDEs). The possibility that Nip1 may have a PDE-like activity remains to be investigated. The presence of such an activity would be important for intracellular signaling in response to cell death–inducing stimuli.

The subcellular localization of Nip3 appears to be different from Nip1 and Nip2 and resembles more closely a mitochondrial staining pattern (see Alberts et al., 1989). Preliminary immunofluorescence studies using mitochondrial-specific dye rhodamine 123 (Summerhayes et al., 1982) also suggest mitochondrial localization of Nip3 (data not shown). In cells coexpressing the 19 kDa protein and Nip3, the localization pattern of Nip3 is altered. The expression of the 19 kDa protein may sequester Nip3 at the nuclear envelope/endoplasmic reticulum regions, thereby abrogating a mitochondrial function of Nip3. It is widely accepted that mitochondria are the high capacity, low affinity stores of intracellular Ca^{2+}. In contrast, the endoplasmic reticulum functions as a low capacity, high affinity store of releasable Ca^{2+} (Berridge and Irvine, 1989). The expression of Bcl-2 protein in certain hematopoietic cells has been shown to regulate the intracellular pools of Ca^{2+} by interfering with repartitioning of Ca^{2+} between the nonmitochondrial (i.e., primarily the endoplasmic reticulum) and mitochondrial stores (Baffy et al., 1993). Similarly, the 19 kDa protein has also been implicated in mobilization of intracellular Ca^{2+} (Subramanian et al., 1985). Nip3 may play a role in repartitioning Ca^{2+} between the two major intracellular Ca^{2+} stores in association with the 19 kDa or Bcl-2 proteins.

Thus, we have identified and cloned multiple cellular proteins that interact with two different proteins (i.e., 19 kDa and Bcl-2) that suppress death induced by a variety of stimuli. Since these interactions occur at domains previously demonstrated to be required for cell death suppression, understanding these interactions and the functions of these cellular proteins will be critical for elucidating the biochemical mechanisms leading to the apoptotic pathway.

Experimental Procedures

Plasmids

Plasmid vectors pMA424 (Ma and Ptashne, 1987) and pAS1 (Durfee et al., 1993) have been described. Plasmids expressing the bait proteins were constructed by cloning the coding sequences of the Ad2 19 kDa protein or the human Bcl-2 protein (Hockenbery et al., 1990) in vectors pMA424 or pAS1. Plasmids pMA-19kDa and pAS-19kDa express residues 2–175 of the 19 kDa protein, and plasmids pMA-Bcl2 and pAS-Bcl2 express residues 1–239 of the Bcl-2 protein. The DNA sequences

coding for the bait proteins were generated by polymerase chain reaction and cloned between the unique EcoRI and BamHI sites of pMA424 and pAS1 (19 kDa) or between the EcoRI and SalI sites (Bcl-2). Similarly, the mutants of *19K* (Subramanian et al., 1993) and *Bcl-2* (T. S. et al., unpublished data) were cloned in the vector pMA424. The cDNA sequences were cloned into the expression vectors pCMV-HA, pTM1 (Moss et al., 1990), and pET3b (Studier et al., 1990), respectively.

Screening cDNA Library

The human (B-cell) cDNA library tagged with the GAL4 activation domain has been described (Durfee et al., 1993). The two-hybrid screens were carried out using the indicator strain GGY1::171 (Gill and Ptashne, 1987) or Y153 (Durfee et al., 1993) by simultaneous transformation with plasmid pMA-19kDa or pAS-19kDa and the cDNA library essentially as described (Chien et al., 1991; Durfee et al., 1993). The interaction of the selected cDNA clones with various heterologous protein baits was then determined to ascertain further the specificity of interaction with the 19 kDa bait.

Protein Interactions

To examine in vitro interactions, ^{35}S-labeled proteins were prepared by in vitro transcription and translation of cDNAs cloned in pET3b vector using a commercially available kit (Promega). These proteins were incubated with unlabeled extracts of HeLa or 293 cells, and the mixtures were subjected to immunoprecipitation with an anti-peptide 19 kDa antibody (gift from M. Green). Extraction and incubations were carried out in 50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% NP-40 with the protease inhibitors leupeptin and aprotinin. The samples were washed at least eight times with the same buffer containing 0.01% NP-40. The labeled proteins were also incubated with extracts prepared from CHO cells or CHO cells infected with an Ad2 recombinant virus expressing the human Bcl-2 protein (Ad–Bcl2) under the transcriptional control of the CMV promoter (T. S. et al., submitted) and immunoprecipitated with a hamster monoclonal antibody (6C8) directed against the human Bcl-2 protein (Hockenbery et al., 1990). The immunoprecipitated proteins were analyzed by SDS–PAGE.

To demonstrate in vivo interactions, the vaccinia virus T7 RNA polymerase hybrid system was used essentially as described by Ausubel et al. (1992). BSC40 cells were infected with vTF7-3, a vaccinia virus expressing T7 RNA polymerase (Fuerst et al., 1986), then cotransfected with pTM1 plasmids expressing one of the HA-tagged Nip proteins and either 19 kDa or Bcl-2, using LipofectAMINE (GIBCO BRL). Cells were labeled with [^{35}S]methionine–cysteine mix (EXPRE^{35}S^{35}S protein labeling mix, New England Nuclear), and lysed 18–20 hr postinfection, as described above. Clarified lysates were subjected to immunoprecipitation using either the 19 kDa antiserum or the monoclonal Bcl-2 antibody 6C8, as well as the HA monoclonal antibody 12CA5. The immunoprecipitated proteins were analyzed by SDS–PAGE to determine the relative levels of the 19 kDa or Bcl-2 proteins present in cells cotransfected with either of these and one of the HA-tagged Nip proteins or the HA vector. The HA immunoprecipitates were separated by SDS–PAGE, blotted, and probed with the appropriate antibodies against 19 kDa or Bcl-2. Horseradish peroxidase–coupled secondary antibodies were used with the ECL detection system (Amersham) to visualize antibodies bound to protein on the blots. Exposures averaging 20 s were required to record images on X-ray film.

DNA Sequence Analysis

DNA sequences of the various cDNA clones were determined directly from pACT-based plasmids containing the cDNAs or after subcloning into pBluescript II KS(+) vector by the dideoxy chain termination method using Sequenase version 2 (United States Biochemical Corporation). Sequences upstream of those present in the isolated cDNA clones were obtained by 5′ RACE (Frohman et al., 1988) using a commercially available kit (5′-AmpliFINDER, Clontech) or from cDNA clones isolated (Ausubel et al., 1992) from a λZAP human B cell cDNA library. Downstream sequences were obtained by 3′ RACE using a commercially available kit (GIBCO BRL).

Immunofluorescence Analysis

The cDNAs were cloned into the expression plasmid pCMV-HA. COS7 cells, grown on 22 mm^2 coverslips in 30 mm dishes, were transfected with plasmids expressing the 19 kDa protein (pRcCMV-19kDa), or each of the pCMV–HA cDNA clones, or both, using LipofectAMINE. Cells were fixed with 3.7% formaldehyde in PBS 48 hr posttransfection and permeabilized with methanol. Cells were double stained with mouse monoclonal HA antibody and rabbit polyclonal 19 kDa antipeptide serum and visualized with goat anti-rabbit rhodamine conjugate (Cappel) and goat anti-mouse fluorescein conjugate (Pierce). Cells were observed and photographed for rhodamine (19 kDa) and fluorescein (HA-tagged proteins) fluorescence.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are as follows: *Nip1*, U15172; *Nip2*, U15173; *Nip3*, U15174.